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EFFECTS OF THE AFFINITY LIGANDS 14-β-CHLOROACETYLNALTREXONE AND 14-β-BROMOACETAMIDOMORPHINE ON [^{3}H]-DIHYDROMORPHINE BINDING SITES IN RAT BRAIN*

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Abstract—The aim of the present study was to examine the inhibitory effects *in vitro* of the affinity ligands 14-β-chloroacetylnaltrexone (CAN) and 14-β-bromoacetamidomorphine (BAM) to characterize the pharmacological specificity of the ligands for high and low affinity opioid binding sites. Rat brain membranes were incubated with 2.0 μM BAM or CAN, or their parent compounds (morphine and naltrexone, respectively) at 37° for 45 min, and the membranes were washed extensively to remove the unbound ligand. The specific binding of 0.3 nM [^{3}H]dihydromorphine ([^{3}H]DHM) was reduced 32 ±7% in membranes treated with CAN and BAM, whereas specific binding in preparations treated with morphine and naltrexone was not significantly different from controls. An increased affinity of BAM and CAN relative to morphine and naltrexone could not account for the observed irreversible inhibition, since the relative affinity of CAN was similar to that of naltrexone and that of BAM was 10-fold less active than morphine. Saturation binding assays revealed that the affinity ligands selectively abolished a high affinity binding site (K_d = 0.3 nM, B_{max} 95 fmoles/mg protein), which comprised approximately one-third of the total number of sites. The affinity of the remaining site (K_d = 4.0 nM) was not altered significantly. The results indicate that the inhibition caused by the affinity ligands is irreversible and represents inactivation of high affinity opioid binding sites in a relatively selective manner.

Recently, two novel opioid affinity ligands, 14-β-chloroacetylnaltrexone (CAN) and 14-β-bromo acetamidomorphine (BAM), have been used to label and purify active opioid binding proteins from rat brain [1–3]. Both CAN and BAM were shown to react with approximately one-third to one-half of the total number of [^{3}H]dihydromorphine ([^{3}H]DHM) binding sites in neural membranes in an apparently irreversible manner. Exposure of neural membranes to ^{125}I-labeled affinity ligands resulted in the labeling of three proteins corresponding to those obtained from affinity gels prepared with BAM and CAN [1, 2]. Affinity chromatography, using BAM coupled to aminohexyl-sepharose, afforded a 2000-fold purification of the opioid receptor complex, which consisted mainly of three proteins with molecular weights of 23, 35 and 45 kD. The relative affinity of opioids for the purified material was similar to the affinity of the receptors for membranes, although the B_{max} of the purified preparation was lower than expected.

Although the results demonstrate that BAM and CAN react covalently, details on the binding characteristics of CAN and BAM are lacking, particularly concerning their interaction with high affinity binding sites. Pasternak *et al.* [4] have proposed that the high affinity component (μ_1) of the binding sites for morphine and enkephalin, as determined by Scatchard and displacement plots of opioid binding, represents an opioid receptor subtype that is involved in the analgesic response elicited by various opiates. The aim of the present study was to examine the inhibitory effects, *in vitro*, of CAN and BAM with a view to determining if the ligands selectively inactivated the high affinity μ_1 site.

METHODS

Materials. [^{3}H]Dihydromorphine (sp. act 75–80 Ci/mmole) was purchased from New England Nuclear (Boston, MA) and morphine sulfate was obtained from Mallinckrodt (Paris, KY). The synthesis of BAM and CAN has been described in detail elsewhere [2, 3]. The "Ligand" software package (Med. 58) was obtained from the BMCC at the University of Tennessee (Memphis, TN). The rats used were Sprague–Dawley males weighing 200–300 g.

Rat brain membranes were prepared by rapidly homogenizing the brain of a rat (minus the cerebellum) in 20 vol. of ice-cold Tris buffer (50 mM, pH 7.7, at 25°) with a polytron for 30 sec at setting 5.5. The membrane suspension was centrifuged at 30,000 *g* for 20 min, resuspended in the original volume of buffer, incubated for 45 min at 37° and centrifuged. The pellets were resuspended to a final volume of 70 ml, yielding a final protein concentration of 1.1 mg/ml (±0.1).

Treatment with BAM and CAN. Membrane prep-

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arations (10–35 ml) were incubated for 45 min at 25° with or without 2 μM BAM or CAN or 2 μM morphine or naltrexone. After centrifugation, the pellets were washed four additional times by resuspension of the pellets in 100 vol. of buffer followed by centrifugation at 30,000 *g* for 30 min. After the fourth wash, the membranes were incubated for 45 min at 25° to dissociate reversibly bound ligand. The membranes were then centrifuged and resuspended to the original tissue dilution for the measurement of [^{3}H]DHM binding.

Measurement of [^{3}H]DHM binding. The procedure was based on that of Pasternak and Snyder [5]. For Scatchard analysis, triplicate sets of tubes containing 0.2 to 20 nM [^{3}H]DHM with or without 2 μM morphine or naltrexone + 1.0 mg membrane protein (treated as described above) in a final volume of 0.5 to 1.0 ml of 0.05 M Tris, pH 7.5, were incubated for 1 hr at 25°. [^{3}H]DHM binding was also measured at various concentrations of morphine, naltrexone, BAM, and CAN. At the end of the incubation, the preparations were filtered *in vacuo* through Whatman GF/B filters and washed twice with 5 ml of ice-cold buffer; the filters were counted by liquid scintillation. Binding parameters were obtained through the use of computerized, weighted, non-linear least squares regression (the "Ligand" software package). Student's *t*-test or the F test was used for statistical analysis.

RESULTS

Effect of CAN or BAM on [^{3}H]DHM binding compared with morphine and naltrexone. Without washing membrane pellets exposed to various concentrations of competing ligands, a comparison was made of the abilities of BAM and CAN with that of their respective parent compounds, morphine and naltrexone, to compete for [^{3}H]DHM binding (Fig. 1). The effectiveness of BAM was about one-tenth of that of morphine, whereas that of CAN and of naltrexone were virtually identical. Specific [^{3}H]DHM binding of membrane preparations that were washed five times after treatment with 2 μM BAM or CAN was inhibited, on the average, approximately 32 and 38%, respectively, compared with untreated controls (Fig. 2). Specific binding after treatment with the 2 μM morphine and naltrexone did not differ significantly from that of untreated preparations. The relative affinities of BAM and CAN for [^{3}H]DHM binding sites were compared with their parent compounds to determine if the apparent irreversible effect could be due to a difference in the relative affinities of the two ligands compared with the parent opiates for [^{3}H]DHM binding sites. The potency of CAN was found to be similar to that of naltrexone, while BAM was approximately 10-fold less potent than morphine (Fig. 2).

Scatchard plots of [^{3}H]DHM binding in membranes treated with BAM or CAN. Specific [^{3}H]DHM binding was linear with protein concentration (0.25 to 1.5 mg/ml) and was saturable, while non-specific binding was linear with radioligand concentration. Specific binding accounted for around 60–90% of the total bound over radioligand concentrations ranging between 0.2 and 8.0 nM (data not shown). Scatchard transformations of the saturation curves were curvilinear in untreated membranes (Fig. 3). The data were analyzed using the "Ligand" program, and a two-site model fit the data significantly better than a one-site model ($P < 0.05$ using F tests). The parameters of the high affinity site were $K_H = 0.32$ nM, $B_{max} = 0.095$ pmole/mg protein, and a lower affinity site with $K_L = 4.11$ nM, $B_{max} = 0.235$ pmole/mg protein.

Scatchard analyses on [^{3}H]DHM binding were performed after exposure of membrane preparations to either BAM or CAN (Figs. 3 and 4). Compared with the respective parent compounds, BAM and CAN selectively inhibited the high affinity binding

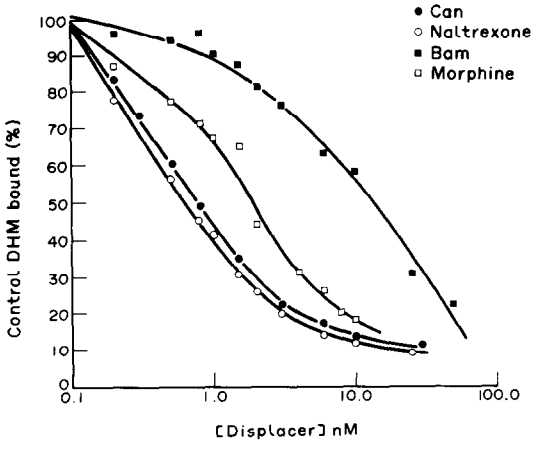

Fig. 1. Competition of various concentrations of BAM, CAN, and their parent ligands for [^{3}H]DHM binding. Membranes were incubated with 0.9 nM [^{3}H]DHM in the presence of various concentrations of unlabeled ligands. Each point represents the mean from two separate experiments with an average coefficient of variation under 10%.

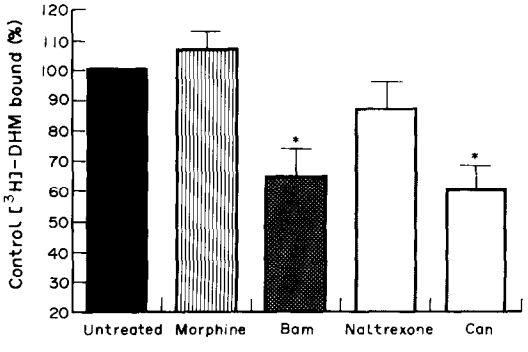

Fig. 2. Effects of CAN and BAM on [^{3}H]DHM binding compared with morphine and naltrexone. Neural membranes were incubated for 45 min at 37° with 2.0 μM BAM or CAN, or their parent compounds, and then washed five times, as described in Methods, and specific [^{3}H]DHM binding was determined. Values are expressed as the percentage of the specific binding in untreated membranes that were carried through the washing procedure. The values represent the means (±S.E.) from three experiments (*$P < 0.01$ compared to controls).

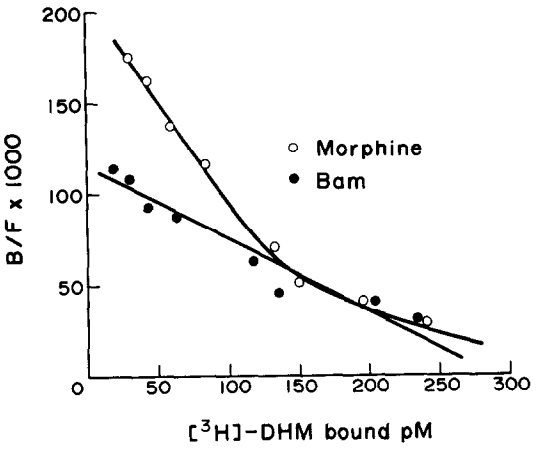

Fig. 3. Scatchard plots of [^{3}H]DHM specific binding in membranes treated with BAM or morphine. Membranes were incubated with a 2 μM concentration of the opiates for 45 min at 37° and washed five times. Specific binding of 0.2 to 8 nM [^{3}H]DHM was determined as described in the text. The values are the means from two experiments.

component observed in untreated membranes and in membranes treated with a similar concentration of either morphine or naltrexone. Analysis of the data with the "Ligand" program revealed that the morphine treatment did not alter opioid binding significantly; after the five washes, both high and low affinity sites were still apparent, with the high affinity site comprising 36% of the total number of sites ($\alpha = 0.36$). BAM abolished high affinity [^{3}H]DHM binding, but did not alter the K_d, nor affect significantly the total number of binding sites (Fig. 3). Similarly, naltrexone treatment did not affect the relative proportion of high to low affinity binding sites ($\alpha = 0.36$), while the high affinity site was not present after CAN treatment, with little change in the B_{max} compared with naltrexone-treated samples (Fig. 4).

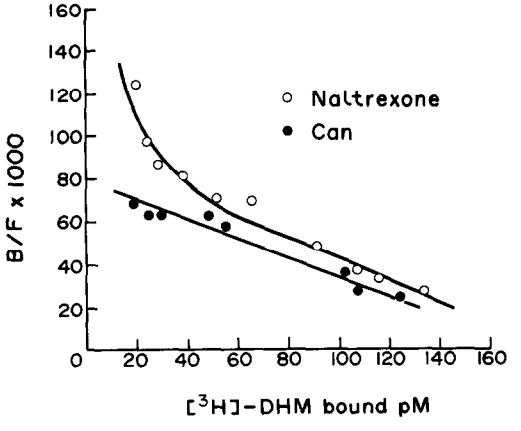

Fig. 4. Scatchard plots of [^{3}H]DHM specific binding in membranes treated with CAN or naltrexone. Neural membranes were incubated with 2 μM CAN or naltrexone for 45 min at 37° and washed five times. The values are the means from three experiments that had an average coefficient of variation of under 10%.

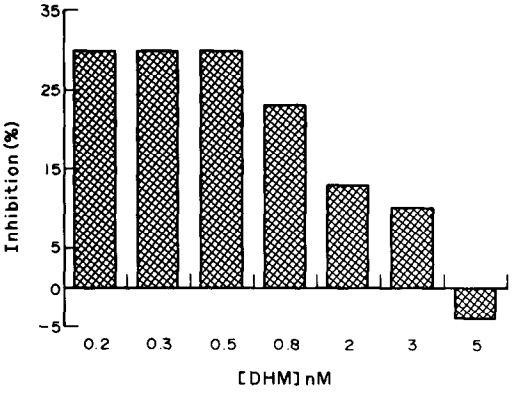

Fig. 5. Inhibition of [^{3}H]DHM specific binding by BAM relative to morphine. Membranes were treated with 2 μM BAM or morphine and washed five times as described in Methods. Values, which represent the ratio of the percent inhibition by BAM to that by morphine, are an average of two experiments (in triplicate) with the coefficient of variation less than 8%.

Figures 5 and 6 illustrate the relative inhibition of DHM binding by CAN and BAM compared to their respective parent compounds, at various concentrations of [^{3}H]DHM. The inhibitory effects of the affinity ligands diminished with increasing concentration of [^{3}H]DHM, as would be predicted if the ligands selectively inhibit the high affinity binding site. If both sites were affected equally, simple (empirical) modeling predicts that the level of inhibition would be the same across [^{3}H]DHM concentration. It can also be seen that, at higher concentrations of DHM, the inhibiting effect of both affinity ligands relative to the parent compounds decreases.

DISCUSSION

The present study confirms previous findings [1, 2] that BAM and CAN will irreversibly inhibit opioid binding to rat brain membranes. It has also been demonstrated that the high affinity component was completely and irreversibly inhibited by both

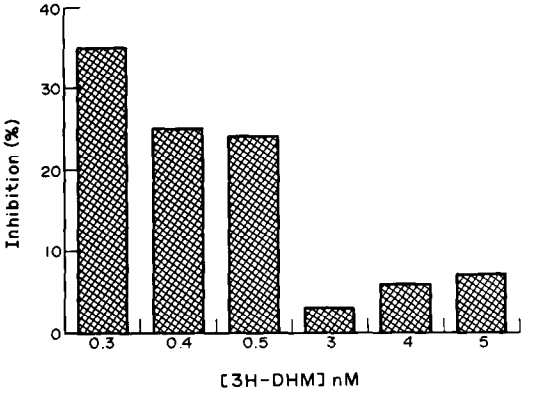

Fig. 6. Inhibition of [^{3}H]DHM specific binding by CAN relative to naltrexone. Conditions were as in Fig. 3 except that 2 μM CAN and naltrexone was used.

ligands, with no effect of BAM and a slight increase with CAN on the K_d of the lower affinity site. The selectivity of the ligands for the high affinity site was borne out by both the Scatchard curves and the relative inhibition at [^{3}H]DHM concentration near the K_d values of the high and low affinity sites. The latter type of analysis provides more compelling evidence for selective inhibition by avoiding pitfalls in interpreting curvilinear Scatchard plots [6].

The properties of BAM and CAN are similar to those of the opiate antagonist, naloxazone [5], which abolished the high affinity binding of [^{3}H]DHM and [^{3}H]DADLE with little or no effect on the δ and lower affinity μ site [3]. Insofar as only the high affinity μ site (designated μ_1) was inhibited *in vivo* by a dose of naloxazone inhibiting morphine-induced analgesia, it was postulated that the μ_1 component was specifically related to analgesia.

In a series of naltrexone-derived affinity labels examining the importance of C-6 chirality in conferring irreversible antagonism, it was reported that β-funaltrexamine was selective for the μ-receptor [7, 8]; on the other hand, the more reactive β-chlornaltrexamine, which irreversibly inhibited [^{3}H]naloxone binding to brain membranes 40% and increased the ED_{50} of morphine-induced analgesia, was not selective for any opiate subtype [8]. It has been reported that fenatnyl isocyanate irreversibly inactivates the δ-site in NG108-15 neuroblastoma × glioma hybrid cells, but no data are available on the specificity of this ligand for subtypes [9]. Insofar as isocyanate is a highly reactive electrophile, while naltrexamine isocyanates have been reported to be non-selective [8], the fentanyl analogue is not expected to be selective.

In conclusion, the present study has demonstrated that opiate affinity ligands with electrophiles at the 14-β-position of heterocyclic ring exhibit a specificity for the μ-subtype, particularly the high affinity component. Furthermore, the antagonist and agonist ligands appear to act similarly in their interaction with the recognition site. The findings also support the concept that less reactive electrophiles, such as the bromoacetamido and chloroacetyl groups, impart greater receptor selectivity to opiate affinity ligands [8].

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